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DNA Fingerprinting Reveals a Lack of Genetic Variation in Northern Populations of the Western Pond Turtle (*Clemmys marmorata*)

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Abstract: *I used DNA fingerprinting to provide the first analysis of the genetic composition of western pond turtle (*Clemmys marmorata*) populations in Washington, Oregon, and California. Populations of the western pond turtle in Washington and northern Oregon are rapidly approaching extinction. Genetic similarity within the largest northern populations, which are located inland, is high. An analysis of population substructure (F_{st}) revealed significant genetic divergence between inland populations, indicating a lack of dispersal and gene flow between sites. In contrast, northern coastal sites are not genetically distinct, but there are few if any viable populations remaining in this region. Genetic variability within southern California populations is a great deal higher than in northern inland sites. Similarly, a low F_{st} value indicated a lack of genetic differentiation between southern sites. An inter-regional analysis of population substructure (F_{st} = 0.24) revealed a significant degree of genetic divergence between geographical regions throughout the range. In addition, an estimate of western pond turtle phylogeny showed a genetic break in the species between northern and southern populations. Both population subdivision and phylogenetic analyses suggest a lack of appreciable gene flow between geographical regions for a considerable period of time. Genetic analyses support traditional subdivision based solely on the morphological variation of* Clemmys marmorata *into two subspecies: northern* Clemmys marmorata marmorata *and southern* Clemmys marmorata pallida. *Recovery of dwindling northern populations must combine demographic and genetic considerations. A first step should be to preserve local gene pools while augmenting population numbers, with the goal of preventing the extinction of this genetically and morphologically distinct subspecies.*

La falta de variación genetica en poblaciones norteñas de la tortuga de agua dulce del oeste (*Clemmys marmorata*)

Resumen: *En este estudio se utilizó análisis de "huellas digitales" de ADN para presentar el primer análisis sobre composición genética en poblaciones de la tortuga de agua dulce del oeste (*Clemmys marmorata*) en Washington, Oregon y California. Las poblaciones de esta tortuga en los estados de Washington y Oregon están al borde de la extinción. La similitud genética dentro de las poblaciones norteñas más grandes, que están localizades tierra adentro, es alta. Un análisis de a subestructura de los poblaciones (F_{st}) muestra una divergencia genética significativa entre las poblaciones del interior, lo que indica una ausencia de dispersión y flujo génico entre ellas. En contraste, los sitios costeros del norte no son genéticamente distintos, sin embargo, en esta región las poblaciones viables que quedan son pocas o inexistentes. Las poblaciones del sur de California presentan una variabilidad genética mucho mayor que las poblaciones norteñas del interior. Para las localidades del sur se obtuvieron valores bajos de F_{st}, lo que indica que no hay diferenciación genética entre estas poblaciones. También se realizó un análisis inter-regional de subestructura poblacional, y se encontró una divergencia genética significativa entre las regiones geográficas a lo largo del rango estudiado (F_{st} = 0.24). Adicionalmente, una estimación de la filogenia de* C. marmorata *mostró una discontinuidad genética entre las poblaciones del norte y del sur. Tanto el análisis de subdivisión poblacional como el análisis filogenético sugieren que no ha habido flujo génico apreciable entre las regiones geográficas desde hace bastante*

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tiempo. El análisis genético apoya la subdivisión tradicional, que se basa únicamente en la variación morfológica de Clemmys marmorata, *y la separa en dos subespecies:* Clemmys marmorata marmorata *en el norte y* Clemmys marmorata pallida *en el sur. Para la recuperación de las poblaciones menguantes del norte se tendrán que considerar tanto los aspectos geográficos como los genéticos. Un primer paso sería preservar los bagajes génicos locales a la vez que incrementar dichas poblaciones, con el objetivo de prevenir la extinción de estas subespecies que son diferentes tanto morfológica como genéticamente.*

Introduction

Populations of the western pond turtle (*Clemmys marmorata*), a freshwater species native to the west coast of the United States, are declining throughout its range. Historically, the western pond turtle inhabited aquatic ecosystems from the southwestern portion of British Columbia to northwest Baja California, west of the Cascade-Sierran crest (Stebbins 1966; Bury 1970). Since the 1800s, population numbers have decreased rapidly, especially in the northern portion of the range, due to habitat alteration, human disturbance, disease, and the introduction of predators such as largemouth bass (*Micropterus salmoides*) and bullfrogs (*Rana catesbeiana*) (Milner 1986). Currently, the western pond turtle is extinct in British Columbia (Holland 1991*b*), listed as endangered in Washington state (Washington Department of Wildlife 1991), sensitive with critical standing in Oregon (Holland 1992), and a species of special concern in California (Steinhart 1990). According to current censuses, fewer than 100 wild individuals remain in Washington (Nordby 1992), and Holland (1991*a*) has predicted that, without formal management, northern populations of western pond turtles may become extinct in as few as 10 to 15 years.

Western pond turtles inhabit a variety of aquatic habitats, including rivers, streams, lakes, ponds, and wetlands (Holland 1991*b*). Individuals range in size from a carapace length approximately 25 mm upon hatching to a carapace length of 120 to 200 mm as reproductive adults. Females begin breeding between eight and 14 years of age and reach sexual maturity earlier in the more southern part of their range. It is estimated that females oviposit in alternate years, laying an average of six eggs (range 1–13) per clutch (Holland 1992). Nests are typically located between 12 and 75 meters from water (Holland 1991*a*, 1991*b*), and a male-biased adult sex ratio is probably the result of predation on females while nesting. Hatchling survival is much lower (8–12%) than yearly adult survival rates (45%). The potential lifespan of an adult is approximately 50 years (Holland 1991*b*).

The rapid decline of the western pond turtle has generated serious concern over both its demographic and its genetic viability. Demographic data collected since 1984 indicate that the majority of western pond turtle populations in Washington and Oregon appear to lack sufficient juvenile recruitment for population maintenance (Zimmerman 1986; Holland 1992; Nordby 1992). A similar lack of juvenile recruitment in other turtle species has been shown to cause unstable population fluctuations and an increased probability of extinction (Congdon et al. 1993). Due to the increasing isolation of local populations, successful overland dispersal in northern populations is rare, essentially eliminating immigration and emigration in these regions (Holland 1991*b*). A similar lack of movement between populations despite long-distance travel to nesting sites is also seen in the wood turtle (*Clemmys insculpta*; Quinn & Tate 1991). Such small and isolated populations are more prone to extinction due to poor habitat quality, fluctuating environments, and increased difficulties finding mates (Lande 1988). In addition, these fragmented and isolated populations may suffer from inbreeding and loss of genetic variation, which are known to reduce the fitness of many animals (Watt 1983; O'Brien et al. 1985). Specifically, inbreeding often increases the proportion of deleterious recessives throughout an individual's genome (Packer 1979), increasing risk of extinction (O'Brien et al. 1986) by causing a reduction in viability and fecundity (Wright 1977; Ralls & Ballou 1983; Sausman 1984; Templeton & Read 1984; Ralls et al. 1986) and a reduction in litter size (Wright 1977). Likewise, loss of genetic variation may reduce chances of adapting to a changing environment (Lande 1988) and increase susceptibility to disease (O'Brien et al. 1985).

Although census and basic life-history data have been collected for the western pond turtle, nothing is known about genetic differences between populations. For example, do different populations represent one large, highly fragmented but genetically similar unit, or do specific ponds or drainages represent distinct, highly inbred populations? In this study, I generated data on levels of genetic variation both within sites and within and between the geographical regions represented by sites inhabited by the western pond turtle throughout its range.

I used DNA fingerprinting to examine the genetic structure of western pond turtle populations in Washington, Oregon, and California. DNA fingerprinting is commonly used as an index of genetic diversity (Wetton et al. 1987; Jeffreys et al. 1988; Gilbert et al. 1990; Lynch 1990; Reeve et al. 1990; Meyer et al. 1991; Wayne et al. 1991; Neuhaus et al. 1993). Fingerprinting is based on

the discovery that nuclear genomic DNA has hypervariable minisatelite regions—regions of the DNA composed of tandem repetitive units of a common core sequence. The number of repeats in each region is so variable that individuals are typically heterozygous at almost every locus (Jeffreys et al. 1985; Burke & Bruford 1987; Wetton et al. 1987). Hybridizing DNA fragments with probes for these minisatellite core sequences generates "fingerprints" that are highly specific to individuals. Because DNA fingerprinting data are based on information from a large number of independent and biparentally inherited loci, this technique enables researchers to assay much more genetic variation than other molecular techniques (Loukas et al. 1986; Anderson et al. 1987; Quinn & White 1987; Westneat 1990). In some cases, the application of fingerprinting may be limited because specific allelic states cannot be assigned to individual loci. DNA fingerprinting, however, may be more preferable than the conventional allozyme method for estimating diversity, which can be plagued by a limited number of detectable polymorphisms in some taxa (Barrowclough 1983; Sherwin et al. 1991; Goodwin et al. 1992; Karl et al. 1992; Neuhaus et al. 1993). Similarly, there is unusually low genetic variability among mtDNA lineages in Testudines (Avise et al. 1992). In a mtDNA survey of 59 restriction sites, Avise et al. (1992) found no restriction-site variation either within or among sampled populations of the map turtle (*Graptemys geographica*); a similar low level of genetic diversity among mtDNA lineages was detected in five other turtle species as well. As a result fingerprinting may be more useful for determining relationships between populations that have been separated by recent habitat fragmentation and isolation, as is the case with the western pond turtle. Hypervariable minisatellite regions of DNA have very high mutation rates (Jeffreys et al. 1988) and therefore are highly sensitive in detecting differences between related genomes (Goodwin et al. 1992; Neuhaus et al. 1993). As such fingerprinting can reveal differences between populations or other groups that have been isolated for only a short period in evolutionary time—several hundred years or less (Gilbert et al. 1990).

DNA fingerprinting is widely recognized as a useful tool for assessing intra- and interpopulation genetic diversity (Faulkes et al. 1990; Lynch 1990; Meyer et al. 1991; Neuhaus et al. 1993). Within a single population, one can determine how genetically similar individuals are to one another (Kuhnlein et al. 1989). This measure then can be compared to the degree of similarity between close relatives to assess the degree of within-population genetic variation. By pooling population data, one also can determine the level of genetic similarity among individuals within a particular locale or geographical region (Faulkes et al. 1990; Lynch 1990; Wayne et al. 1991). And finally, one can estimate historical and evolutionary relationships between populations using genetic distances generated from fingerprinting data (Nei & Li 1979; Felsenstein 1984; Anderson et al. 1987; Swofford & Olsen 1990).

My study addresses four main questions: (1) How genetically similar are western pond turtles within each study site? (2) How does within-site similarity compare with the degree of genetic similarity between related individuals? Specifically, is there a loss of genetic variation within sites? (3) How genetically similar are western pond turtles within and between three geographical regions: the Columbia River Gorge region of Washington and Oregon, the coastal basins of the Pacific Northwest, and southern California? (4) What is the phylogenetic relationship between the study sites?

Methods

Blood Collection

Blood samples from 80 individuals were collected from four sites in Washington, two in Oregon, and three in California to ensure a wide range of genotypes throughout the western pond turtle's range (Table 1). Sixty-five of these individuals were unrelated and 15 were siblings. Sites 1, 2, 3, and 5 are in the Columbia River Gorge area of Washington and Oregon, sites 4 and 6 represent coastal basins of the Pacific Northwest (the Puget Sound basin and the Willamette River basin, respectively), and sites 7, 8, and 9 are in San Bernardino county, southern California (Fig. 1). Ideally, each site corresponded to a specific pond or drainage system. Because populations in Washington and Oregon are approaching extinction, and most current sitings consist of single individuals (Holland 1992; Nordby 1992), I combined individuals from several drainage systems within a coastal water basin to obtain a small representative sample from the area. I do not know whether the captive group from site 9 originated from a single drainage system; therefore, I chose to be conservative in assigning each to a different drainage. Due to the general lack of successful overland

Table 1. Number of ponds, western pond turtles, and siblings sampled per site.

State	*Location (Site)*	*Number of Ponds or Drainages*	*Total Number of Turtles Sampled*	*Number of Siblings Sampled*
Washington	1	1	6	0
	2	1	2	0
	3	1	13	3
	4	3	4	0
Oregon	5	1	15	0
	6	3	5	0
California	7*	1	8	2
	8	1	21	10
	9*	3	3	0

**Captive population.*

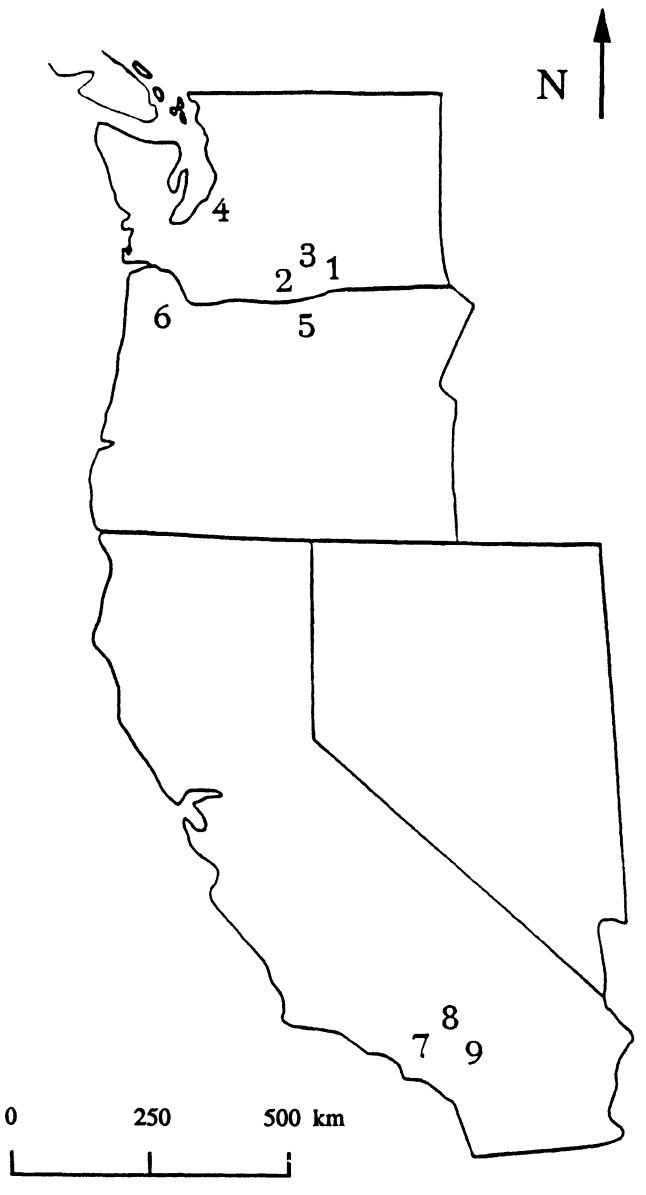

Figure 1. Map of the nine western pond turtle study sites in the western United States.

movement between waterways, I considered each site a distinct population in this study.

Blood was collected from 15 siblings from four clutches: one from Washington, site 3; one from California, site 7; and two from California, site 8. Site 3 siblings were caught as they emerged from their nest; site 8 siblings hatched in captivity after gravid females were captured from the wild and held until their clutches were laid. Siblings from both sites are believed to be full siblings. No incidences of multiple matings have been reported for western pond turtles, but whether these siblings could have been sired by multiple males remains a possibility until paternity analyses are performed on this species. Siblings from site 7 are known full siblings because this captive population has only one breeding male. Because I have no evidence to the contrary, I refer to all siblings as full siblings. Therefore, my comparisons between first-degree relatives include both sibling-sibling and mother-offspring relationships. Nuclear DNA was extracted for each individual from a small quantity of blood (approximately 100 μl). Individuals were bled from the caudal or jugular vein using standard techniques (Avery & Vitt 1984). Mothers of the three California clutches also were bled. Immediately after collection, blood was suspended in a TNE buffer (50:50) and stored at −80°C.

Laboratory Techniques

DNA extraction followed the protocol described by Westneat (1990, 1993). In many cases more than 50 μl of blood was needed to obtain a sufficient quantity of DNA. Because this quantity was not always available, DNA was extracted successfully from only 77 out of the 80 turtles sampled.

Initially a variety of restriction enzymes (*Alu*I, *Hae*III, *Hinf*I, and *Rsa*I), which cleave DNA, were tested to determine which produced the greatest number of distinguishable bands over a wide range of fragment sizes. After this preliminary screening 15 μg of extracted DNA per individual was isolated and restriction-digested with the 4-base cutter endonuclease *Alu* I. Restriction-digested fragments were separated according to size in 0.8% agarose electrophoretic gels (20 × 25 cm gel molds and buffer tanks). The total amount of digested DNA was quantified using spectrophotometry and was adjusted to the same concentration for all lanes on a gel (ranging from 6 to 8 μg of digested DNA per gel). To aid in scoring, all lanes also were loaded with an internal standard 1 Kb DNA ladder (Gibco BRL). Gels were run for 1750 volt hours (48 hours) in a recirculating 1X TBE buffer at room temperature. After each run, the quality of digestion was checked under UV illumination after the gel was stained with ethidium bromide. DNA then was denatured by two washes in 1.5 *M* NaCl, 0.5 *M* NaOH, neutralized by two washes of 1 *M* ammonium-acetate, 0.04 *M* NaOH, and transferred to a nylon membrane (Zetabind, AMF Cuno) in 1 *M* ammonium-acetate, 0.04 *M* NaOH using a vacublot procedure (Westneat et al. 1988). After DNA transfer, membranes were immediately dried and baked.

Hybridization was begun by placing up to six membranes in rotating plexiglass tubes for 48 hours with 30 ml of Na_2HPO_4 prehybridization solution (Westneat et al. 1988). Probe DNA (50–200 ng) was labeled with [^{32}P]dCPT by random priming, and unincorporated nucleotides were removed with columns of Sephadix G-50 beads in TE. Hybridization occurred at 60°C for 48 hours in fresh prehybridization solution. Membranes were radioactively probed with both M13 and Jeffreys 33.6 to obtain a large number of scorable bands per individual

(see Westneat 1993 for details of isolating probes). Washes differed depending on the probe used. Filters probed with both M13 and Jeffreys 33.6 were washed in 2 × SSC and 0.1% SDS twice at room temperature and once at 60°C. Filters probed with 33.6 were washed a second time at 60°C and then once in 1 × SSC at 65°C. After washes, membranes were exposed to film (Kodak XAR) for 2 to 4 weeks at −80°C.

Because scoring accuracy declines as the distance between lanes on a gel increases (Westneat 1990, 1993; Piper & Rabenold 1992), and comparisons cannot be made between individuals run on different gels, samples with high yields of extracted DNA were run several times. Placement of individuals on gels was designed to maximize the number of within- and between-site comparisons. Typically, individuals from the same site were grouped in pairs or trios and placed on gels so that groups from different sites alternated. Thus each turtle was compared at least once to another turtle from the same site, as well as at least twice to turtles from a different site. Individuals from sites with small sample sizes (such as site 4) were run on multiple gels between different pairs or trios to increase the number of comparisons made with these sites.

Only fragments between 3.0 and 12.0 kb were scored for each probe. Bands were scored as the same if their centers were within 0.5 mm of each other and there was no more than a two-fold intensity difference between them (Westneat 1990). The number of bands shared between two turtles was calculated for individuals in adjacent lanes and for individuals separated by one and two lanes on a gel (Piper & Rabenold 1992). Thus, each individual was used in at least two comparisons. Similarity (S) between individuals is defined as

$$S(x, y) = 2n_{xy} / n_x + n_y,$$

where n_x is the number of bands for the first individual, n_y is the number of bands for the second individual, and n_{xy} is the number of bands shared by both individuals (Wetton et al. 1987). Thus, similarity increases as values approach 1.0.

Baseline Index of Similarity between Related Individuals

Assessing genetic relatedness with DNA fingerprinting is complicated by the fact that a baseline level of band-sharing exists between unrelated individuals within a population or species (Lynch 1988; Westneat 1990). However, band-sharing scores (i.e., similarity values) for pairs of individuals of known relatedness can be used as a reference point to assess the degree of genetic similarity between pairs of unrelated individuals (Piper & Rabenold 1992). For example, genetic relationships between individuals may be more or less similar than genetic relationships between first-, second-, or third-degree relatives. In this study, mean similarity values were calculated for both sibling-sibling and mother-offspring relationships to generate a baseline index of similarity for first-degree relatives. One potential bias in this baseline is that the majority of related individuals are from Californian populations. An extensive survey of zoos and private owners conducted during this study, however, revealed no known captive breeding populations of northern *C. marmorata*. Furthermore, no successful nests were discovered in the wild, which could have been sampled in addition to the single clutch originating from site 3.

Site and Regional Analyses

A mean within-site similarity value was calculated for each of the nine sites. All similarity values (S_{xx} and S_{xy}) were calculated for each gel and then averaged across gels. Individuals within a particular geographical region, regardless of site or state, then were pooled to generate a mean similarity value for each geographical location: (1) the Columbia River Gorge area of Washington and Oregon, (2) coastal basins of the Pacific Northwest, and (3) San Bernardino county, southern California.

Use of each turtle in at least two scoring dyads created nonindependence of pairwise similarity values. To adjust for this bias, covariance terms were calculated to estimate statistical dependency, and variance terms were corrected accordingly (Lynch 1990). Overall similarity within geographical regions was calculated using Lynch's equation [11] (1990), which measures between-site similarity corrected for within-site similarity.

Estimates of genetic differentiation between populations within a geographical region, as well as between these three regions, were determined with Wright's (1951) F_{st} statistic. To obtain a downwardly biased and hence conservative estimate of subdivision using similarity data, F_{st} was calculated with a modified formula by Lynch (1990; equation [14]). F_{st} values ranged between 0.0 and 1.0, with high F_{st} values indicating a considerable degree of divergence between areas. The statistical significance of F_{st} values was tested by Chi-square analysis (Workman & Niswander 1970; Nei & Chesser 1983). These data were analyzed with a computer program written in ThinkC for Macintosh.

Genetic divergence between the seven wild sites was estimated in a hierarchical cluster analysis by the unweighted pair-group method using arithmetic averages (UPGMA) (Sneath & Sokal 1973). Similarity values between sites were transformed to account for the within-population variation using the formula

$$S = S_{xy} / \sqrt{S_{xx} \times S_{yy}},$$

according to Nei and Li (1979). Similarity values then were converted to distances with the formula distance

$(d) = -\ln S$ (Swofford & Olsen 1990). The phylogeny was generated using a matrix of pairwise distances between all pairs of populations with PHYLIP, a Phylogeny Inference Package (Felsenstein 1993). The PHYLIP program uses the least-squares method of Cavalli-Sforza and Edwards (1967) for fitting trees to distance matrices (Felsenstein 1982, 1992).

Results and Interpretation

The average number of scorable fragments (ranging from 3.0 to 12.0 kb) per individual was typically greater for the Jeffreys 33.6 probe (Jeffreys 33.6: $X = 24.6 \pm 5.7$; M13: $X = 16.7 \pm 5.2$). Similarity values were calculated for each pair on the sum total number of bands per individual and the sum total number of bands shared across both probings. Thus, all similarity values are based on the results of both probes combined.

Genetic Similarity between Close Relatives

In northern populations, limited success in captive breeding and extremely low nesting success in the wild has made a comparison between related and unrelated individuals from northern latitudes essentially impossible. Therefore, all reference to a familial baseline indicates relatedness primarily between individuals from southern populations.

Seventeen estimates of sibling-sibling comparisons generated a mean similarity value of 0.589 (standard error = 0.003), and 12 estimates of mother-offspring comparisons yielded a mean similarity value of 0.552 (standard error = 0.002). A comparison of means revealed no significant difference between the similarity values of known first-degree relatives (mother-offspring pairs) and presumed first-degree relatives (sibling-sibling pairs) (two-tailed t-test, $t = 0.61$, df = 27, $p = 0.55$). Therefore, all comparisons between related individuals are considered representative of first-degree relationships. As such, mean similarity values ranging between 0.552 and 0.589 indicate a genetic relationship equivalent to that between first-order relatives.

Genetic Similarity within Each Site and Geographical Region

Similarity among banding patterns within a population is directly correlated with the amount of genetic variation assumed to exist in the population (Lynch 1990). Therefore, high within-site similarity values indicate low levels of intra-site genetic variation, and vice versa. Similarity

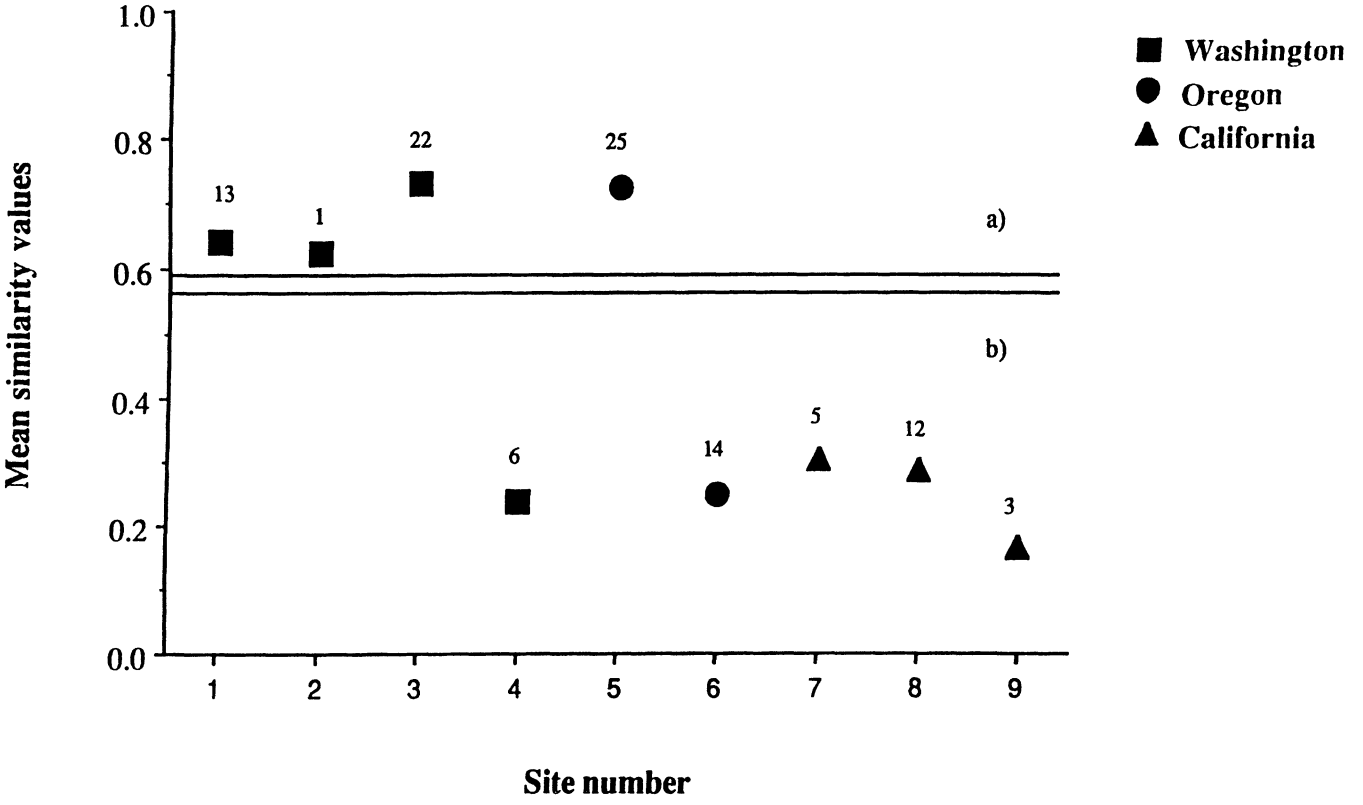

Figure 2. Mean similarity values for each site grouped by state. Upper line (a) is the mean similarity between siblings (a = 0.589; n = 17); lower line (b) is the mean similarity between mothers and offspring (b = 0.552; n = 12). The region between (a) and (b) indicates a genetic relationship equivalent to that of first-degree relatives. The number of pairwise comparisons per site is listed above each data point. All standard errors are ≤ 0.01, except for site 2 (SE = 0.07).

values for each site were plotted as means because all standard errors were less than 0.006 (except site 2 [SE = 0.07] and site 9 [SE = 0.01]).

Mean similarity values within Washington sites 1, 2, and 3 were extremely high (Fig. 2; mean similarity (mean S) >0.60). Individuals within these sites are more genetically similar to one another than individuals in a mother-offspring or sibling-sibling relationship. Thus, based on a comparison with relatives originating primarily from southern populations, turtles from sites 1, 2, and 3 are genetically very uniform. In contrast, Washington site 4 turtles are genetically variable (mean $S <$ 0.25).

The level of genetic variation within site 4 may be overestimated because I could make only a few pairwise comparisons due to the small sample size. More relevant, however, is the fact that individuals from site 4 came from several different drainage systems within the Puget Sound waterbasin. It is possible that individuals from a single drainage system in this area are as genetically uniform as individuals at other Washington sites. Unfortunately, there are insufficient data to make this comparison; the drainage systems I sampled represent multiple genetic groups that are almost extinct.

Results for Oregon follow the same pattern as those from Washington state. Western pond turtles from site 5 are more similar to one another (mean $S > 0.70$) than are full siblings or mothers and their offspring, indicating low levels of genetic variation. In contrast, western pond turtles from site 6 are genetically different (mean $S < 0.25$). Again, the disparity between the two sites may result from the fact that site 6 turtles came from several drainages within the Willamette River basin.

In California genetic variation within sites 7, 8, and 9 is high (mean $S < 0.31$). The level of genetic similarity within all three sites is well below the baseline region of similarity between first-degree relatives, despite the fact that site 7 is a single drainage system and site 8 is a single pond. In contrast to results from Washington and Oregon, therefore, single ponds or drainage systems do not invariably contain genetically depauperate groups of western pond turtles.

Mean similarity values within each geographical region revealed a relatively low degree of genetic similarity between populations in the Columbia River Gorge (mean $S = 0.77$) and high genetic similarity between populations in northwestern coastal basins (mean $S =$ 0.95) and southern California (mean $S = 0.92$; the variance for each region is <0.001). The significance of these values was interpreted with a calculation of population subdivision presented below (similarity = 1-D; Lynch 1990).

Site and Regional Differentiation

Interpopulation genetic differentiation within geographical regions was greater in the Columbia River Gorge than in northwestern coastal regions or southern California (Table 2). Chi-square tests revealed that the only significant population substructure among sites existed in the Columbia River Gorge ($p < 0.001$), indicating a significant lack of gene flow between Gorge sites.

In addition, interregional genetic variability accounted for 24% of the variance observed ($F_{st} = 0.24$), indicating a highly significant degree of genetic differentiation between these three geographical regions ($\chi^2 = 36.34$, $p < 0.001$). This genetic subdivision is consistent with the current morphologically based division of *Clemmys marmorata* into two subspecies: northern *Clemmys marmorata marmorata* and southern *Clemmys marmorata pallida* (Seeliger 1945).

The UPGMA phenogram revealed three separate groupings of *C. marmorata* (Fig. 3). Clustering is inconsistent between states; however, all sites within a major geographical region are more similar to each other than to those of any other region. All Columbia River Gorge sites are similar, despite the fact that site 5 is on the opposite side of the Columbia River from sites 1, 2, and 3. Similarly, sites 4 and 6, both representing coastal basins, are similar despite the approximate 160 km that separate them. Not surprisingly, the greatest difference between sites is between the Washington and Oregon sites and the southern California site. These results, coupled with the analysis of interregional population subdivision and the spatial position of the study sites, indicate that gene flow between northern and southern regions has historically been much less than between populations within the same region.

Discussion

Northern populations of the western pond turtle are rapidly approaching extinction. There are few if any viable populations remaining in northern coastal regions (Milner 1986; Holland 1992; Nordby 1992), and numbers are decreasing rapidly inland as well (Holland 1991*a*). Genetic variation within the largest populations of western pond turtles in northern latitudes, located in the Columbia River Gorge region of Washington and Or-

Table 2. Analysis of population subdivision within and between geographical regions using F_{st} statistics (significance indicated by Chi-square).

Geographical Region	*Sites*	F_{st}	χ^2	*df*
Columbia River Gorge	1, 2, 3, 5	0.244	17.57*	3
Coastal Basins	4, 6	0.047	0.85	1
Southern California	7, 8, 9	0.078	4.99	2
Overall		0.236	36.34*	8

*$p < 0.001$.

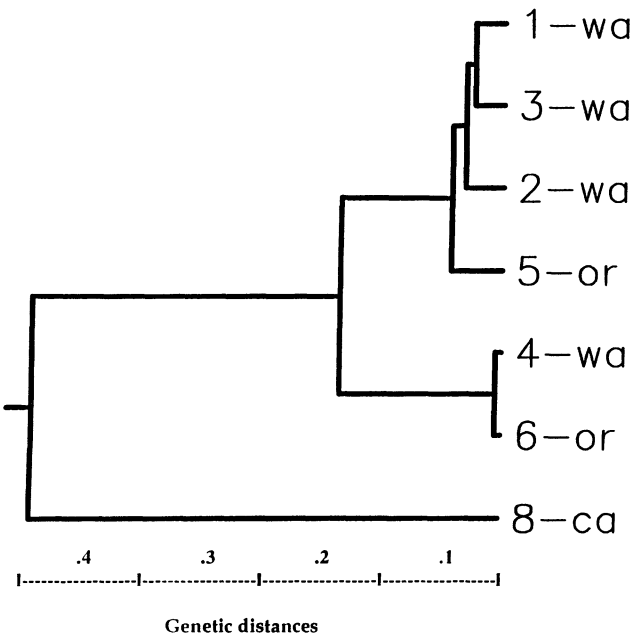

Figure 3. UPGMA phenogram estimating the genetic divergence between the seven wild sites. Sites are abbreviated by number and state: Washington, 1-wa, 2-wa, 3-wa, and 4-wa; Oregon, 4-or and 5-or; California, 8-ca.

egon, is very low. Unrelated individuals within these populations are more genetically similar to one another than first-degree relatives drawn primarily from southern populations. Likewise, an analysis of population substructure revealed significant genetic divergence between Columbia River Gorge sites, confirming a reported lack of dispersal and gene flow between inland areas.

Results also revealed a striking difference between the degree of genetic variability in northern versus southern pond turtle populations. This difference may reflect a history of restricted gene flow in the north; however, there was no genetic subdivision between populations in northern coastal regions, which should exist if gene flow always has been limited between northern sites. Nonetheless, the rapid extinction of western pond turtles in northern latitudes represents a significant loss of genetic variation for the species as a whole.

Lack of Genetic Variation and the Survival of Northern Populations

How genetic homogeneity affects northern pond turtle populations depends on both the extent to which genetic similarity reduces individual survival and fitness and on historical levels of intrasite genetic variation. Genetically similar western pond turtle populations are vulnerable because individuals are less able to adapt to changing environmental conditions, such as continual modification of their habitat for agriculture and urban growth. Equally as important is their increased susceptibility to disease. In the late 1980s, Washington western pond turtles became infected with an upper respiratory disease syndrome known by its acronym URDS, which essentially decimated wild populations by 30% to 40% (Holland 1991*a*, 1992).

Although population genetic data have been collected for a number of turtle species (Seidel et al. 1981; Scribner et al. 1984; McBee et al. 1985; Bonhomme et al. 1987; Bowen et al. 1991, 1992; Karl et al. 1992), no information is available on historic levels of genetic variation in any *Clemmys* species. Because breeding systems or population structure may predispose some species to naturally low levels of genetic heterozygosity within sites (Avise 1989; Scribner et al. 1993), and turtle mtDNA is not highly variable (Avise et al. 1992), low levels of genetic heterozygosity may always have been present in northern populations of the western pond turtle. The extreme difference in the degree of genetic variability between northern and southern populations, however, suggests that genetic homogeneity in *C. marmorata* is a consequence of northern fragmentation and isolation.

It is unlikely that lower genetic diversity in northern populations reflects a recent founder effect. Based on fossil records, ancestors of the western pond turtle probably evolved during the Paleocene period in what is now the central-western portion of the United States (Brattstrom & Sturn 1959). These populations gradually dispersed west, following two separate routes as they colonized the northwestern and southwestern U.S. According to fossil remains, *C. marmorata* had probably colonized most of its current range by the late Pliocene or early Pleistocene (Hay 1903; Brattstrom & Sturn 1959; Gustafson 1973).

Drainage of wetlands and the diversion of water for agriculture consumption and hydroelectric power has essentially removed successful dispersal between northern inland sites. Gene flow along aquatic routes that have since disappeared probably accounts for the high level of genetic similarity within populations in the Columbia River Gorge; without continued gene flow between sites, these differences are expected to increase. Likewise, low population numbers and a lack of dispersal between sites may have limited mate choice, leading to breeding with close relatives. In addition, genetic drift may have decreased genetic variation within sites.

Regional Subdivision

At present, subdivision of the western pond turtle into two subspecies is based solely on morphological variation (Seeliger 1945). Genetic analyses revealed a significant degree of genetic divergence between geographical regions, which is consistent with morphological data. The degree of regional genetic variability (F_{st} = 0.24) found in *C. marmorata* is larger than indices of re-

gional or interpopulation genetic differentiation for other Testudines, such as *Chelonia mydas* (F_{st} = 0.17; Karl et al. 1992) and *Pseudemys scripta* (F_{st} = 0.05; Scribner et al. 1986). In addition, an estimate of western pond turtle phylogeny revealed a genetic break in the species between northern and southern populations. Both population subdivision and phylogenetic analyses indicate spatial heterogeneity and low levels of gene flow between geographical regions, which become more genetically isolated as the distance between them increases. Thus, genetic analyses support the traditional division based exclusively on the morphological variation of *Clemmys marmorata* into the northern subspecies *Clemmys marmorata marmorata* and the southern subspecies *Clemmys marmorata pallida*.

Implications for Management and Recovery

Successful recovery of the western pond turtle in its northern range will depend on a strategy that incorporates knowledge of the genetic composition of existing populations; the ability to maintain suitable habitat for the species, including controlling or eliminating introduced predators; and success in enlarging wild and captive populations to a self-sustaining level. Unfortunately, time is short, and the western pond turtle continues to disappear at an alarming rate. Because many northern populations are small, numbers must be increased if the species is to survive in this region. In addition, recovery efforts must focus on the fact that the largest remaining wild populations in northern latitudes contain little genetic variability. Populations should not, however, be managed with the sole intent of maximizing genetic diversity, especially because there is a significant genetic division between northern and southern populations. Mating genetically dissimilar individuals may disrupt favorable gene combinations, leading to outbreeding depression (Avise 1989). Likewise, increasing genetic variation without consideration of ecological adaptations may lead to a reduction in fitness (Dobzhansky 1948; Cade 1980), which can eventually result in extinction of the species being managed (Greig 1979). Regional hydrography suggests that historically there may have been substantial interchange between coastal regions and the Columbia River Gorge populations via the Columbia River. In contrast, northern populations have probably been well separated from southern California populations since the early Pleistocene. Management efforts should focus on preservation of local gene pools and augmentation of population numbers, perhaps through carefully planned captive breeding and reintroduction or translocation programs, to help prevent the extinction of this genetically and morphologically distinct subspecies.

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